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TMP-REACTIVE AUTOANTIBODIES IN HUMAN SLE SERA DEMONSTRATE THYMINE-DEPENDENT OLIGONUCLEOTIDE SPECIFICITY

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SUMMARY Autoantibodies present in the sera of lupus patients and specific for single-stranded (ss) DNA were fractionated into subsets based upon their reactivity towards 5' nucleotide haptens. As evaluated by ELISA testing, antibodies retained by TMP-agarose bound to TMP-BSA and ssDNA but not to other nucleotide-BSA conjugates or to double-stranded (ds) DNA. Competition-inhibition studies further revealed that TMP-enriched oligo- and polynucleotides were the preferred antigens for these affinity purified antibodies. Similar assays with sequence- or size- defined oligonucleotides further implied that those oligonucleotides comprised entirely of TMP residues were most antigenic and that antigenicity increased with size (length). These results document the existence of a TMP-dependent oligonucleotide specificity among a diverse population of autoanti-ssDNA antibodies.

Autoimmune diseases in general and systemic lupus erythematosus (SLE) in particular are characterized by an abundance of serum autoantibodies that recognize a host of nuclear antigens (1-3). In human and murine lupus, the most abundant autoantibody populations are those reacting with single-stranded DNA (ssDNA) (1-5). These antibodies can be defined in some detail by an enzyme-linked immunoabsorbent assay (ELISA) that employs not only immobilized ss- and dsDNA antigens, but nucleotide haptens as well (5,6). Generally, such haptens are covalently linked to carrier proteins (e.g. BSA), which by themselves are unreactive with autoantibodies. While anti-ssDNA antibodies appear to preferentially recognize individual base moieties within nucleotides (5-8), one cannot dismiss the possibility that sequence-specific (deoxy) oligonucleotides are responsible for antibody interaction with nucleic acids (9-12). For example, several monoclonal anti-ssDNA antibodies have been shown to bind with certain TMP-containing polynucleotides but not others (7-9). More recently, sera preparations from selected autoimmune patients were found to contain autoantibodies specific for the anticodon region of alanine tRNAs (10) and a sequence-specific conformational epitope within U1 RNA

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(11). Other studies from our laboratory have suggested that a significant fraction of anti-ssDNA antibodies in human SLE sera were specific for GMP-enriched oligonucleotides (12). These results prompted us to examine in detail a second nucleotide-reactive autoantibody present in human SLE sera, namely those antibodies reactive with TMP that can be purified free of other serum components by their specific adsorption to TMP-agarose.

METHODS AND MATERIALS Sera were obtained from SLE patients as previously described (12), heat inactivated, and stored at -70°C in the presence of 0.02% azide. All nucleic acids and immunochemical reagents were obtained from Sigma Chemical Company (St. Louis, MO) or from Pharmacia (Piscataway, NJ). Details regarding the synthesis of nucleotide-BSA conjugates and nucleotide-agarose adsorbents have been described (12, 13).

A detailed description of ELISA as it pertains to characterizing anti-DNA autoantibodies has been presented elsewhere (5,12). In all instances absorbance measurements (A405 nm) were determined 30 min after the addition of p-nitrophenylphosphate (alkaline phosphatase substrate) with a Dynatech MR 580 spectrophotometer. ELISA analysis of serially diluted serum revealed that the quantity of antibody bound to immobilized antigen was directly proportional to absorbance values between 0 and 0.70 A405 units. This relationship assumes importance when assessing antibody specificity via competition-inhibition testing (see below).

The utilization of nucleotide-agarose adsorbents for isolating specific autoantibody populations has been documented (12). Briefly, pooled sera (6.0 ml, see Table I) was diluted 2-fold in PBS containing 0.05% Tween -20 and 2 mg/ml BSA (PBS-BT) and incubated with GMP-agarose (2.0 ml) for 2 hr at 24°C. The absorbent was washed via repeated centrifugation-resuspension in PBS-BT before eluting bound antibodies with 2.0 ml aliquots of acidified BSA (1 mg/ml, pH 2.8 to 3.0). For isolation of TMP-reactive antibodies, the first two wash fractions (12.0 ml each) accompanying GMP-agarose adsorption were combined and reincubated with TMP-agarose (3.0 ml). Subsequent procedures (washings, elutions, etc.) were identical to those described above for GMP-agarose. Purified antibodies were neutralized via dilution in PBS-BT prior to storage (4°) and/or further ELISA characterization.

An ELISA competition-inhibition assay was employed to assess the specificity of affinity purified antibodies. These assays consisted of preincubating a variety of soluble competitors (mono-, oligo-, and polynucleotides) with purified antibodies (30 min, 24°C) before ELISA testing. Inhibition of antibody binding to immobilized ssDNA was determined by measuring the ability of each competitor to reduce absorbance (A405) relative to control assays lacking competitor (i.e., % inhibition = [A405(competitor)]/[A405 (control)] x 100; see Figure 1 and Table II). All control assays were within the linear absorbance range as described above.

RESULTS AND DISCUSSION

Anti-DNA Antibodies in Human SLE Sera: Individual and pooled sera from six SLE patients demonstrated a characteristic binding pattern towards nucleotides and nucleic acid antigens when assessed via ELISA.

The data in Table I illustrate this pattern and indicate that those antibodies binding to GMP-BSA and ssDNA are most prominent. While lesser and more variable quantities of immunoglobulins reacted with AMP-, TMP-BSA, and dsDNA, only minimal binding was observed with CMP- and UMP-BSA antigens. What has emerged from these and other ELISA studies is that the distribution of autoantibodies binding to individual antigens appears constant with GMP≅ssDNA>TMP≅dsDNA≅AMP>UMP>CMP.

Affinity Purification of Nucleotide-Reactive Autoantibodies: The binding of autoantibodies not only to DNA but nucleotide haptens as well (Table I) suggested that these populations could be fractionated into subsets based upon their specific adsorption to nucleotide-agarose adsorbents. As depicted in Table I this fractionation scheme appeared successful inasmuch as those antibodies retained by GMP-agarose reacted almost exclusively with GMP-BSA and ssDNA. The slight binding of GMP-reactive antibodies to TMP suggested some cross reactivity with this hapten but not towards other nucleotides or dsDNA. Since the bulk of TMP-reactive antibodies remained in the wash fractions during GMP-agarose adsorption, these fractions were combined and reincubated with TMP-agarose. ELISA analysis of antibodies retained by this second adsorption step revealed their exclusive binding to TMP and to ssDNA (see

Table I. ELISA Characterization of Unfractionated (Serum) and Affinity Purified Autoanti-DNA Antibodies. Assessment of Binding to Nucleotide-BSA Conjugates and to ss- and dsDNA Antigens [a]

Serum No. (Dilution)	Nucleotide-BSA Antigens						DNA	
	BSA	AMP	GMP	CMP	UMP	TMP	SS	ds
	Absorbance (405 nm)							
No. 1 (1:500)	ND[b]	ND	.65	ND	ND	.20	.85	.16
No. 2 (1:500)	ND	.12	.59	ND	.05	.12	.48	ND
No. 3 (1:300)	ND	.06	.44	ND	.08	.30	.75	.08
No. 4 (1:300)	ND	.10	.41	ND	ND	.11	.54	ND
No. 5 (1:300)	ND	.15	.75	ND	.07	.15	.63	.07
No. 6 (1:300)	ND	.20	.48	.05	ND	.12	.53	.17
No. 1-6 (1:400) [c]	ND	.12	.57	ND	ND	.19	.48	.10
Affinity Purified								
GMP-Agarose								
(1:200)	ND	ND	.80	ND	ND	.08	.84	ND
(1:400)	ND	ND	.42	ND	ND	ND	.45	ND
TMP-Agarose								
(1:50)	ND	ND	ND	ND	ND	.78	.80	ND
(1:100)	ND	ND	ND	ND	ND	.42	.39	ND

[a]Antibodies present in the sera of SLE patients were processed via ELISA (Methods Section) as were those affinity purified by GMP- and TMP-Agarose adsorbents. [b]ND: Absorbance Not Detected or less than 0.05 A405 units. [c]No. 1-6, pooled sera.

Table I). These results imply that the majority of nucleotide-reactive autoantibodies are monospecific with respect to a single nucleotide antigen. This assumption is consistent with the finding that these affinity purified antibodies recognize ssDNA (i.e., base determinant exposed) but not dsDNA (i.e., base determinant masked). The inability of both TMP- and GMP- reactive antibodies to recognize other nucleotide hapten (AMP, CMP, UMP) also suggests that the major epitope (antigenic surface) resides within the base structure and not the sugar and/or PO_4 moieties since the latter are common to all nucleotide structures.

Specifity of TMP-Reactive Autoantibodies: A competition-inhibition ELISA was utilized to further assess the specificity of affinity purified TMP-reactive antibodies. As illustrated in Figure 1, only those mono-, oligo- and polynucleotides possessing TMP residues were inhibitory. Also to be noted was the finding that oligo- and polynucleotides enriched with TMP (e.g. poly (dT) versus ssDNA and oligo (dT)6 versus oligo (dN)6) were considerably more potent inhibitors. The difference in inhibition observed between poly (dCdT) and poly (dAdT) was attributed to base-pairing (A:T) in the latter but not the former. Note also that dsDNA and mono- and polynucleotides devoid of TMP were not inhibitory.

TMP-Dependent Oligonucleotide Specificity: Of considerable interest in Figure 1 was the finding that oligo (dT)6 was a superior competitor relative to both TMP and oligo (dN)6. Since the latter was obtained from a DNase I digest of DNA, oligo (dN)6 presumably contains approximately equimolar quantities of all four deoxynucleotides. These data imply, therefore, that oligonucleotide sequences enriched with or containing

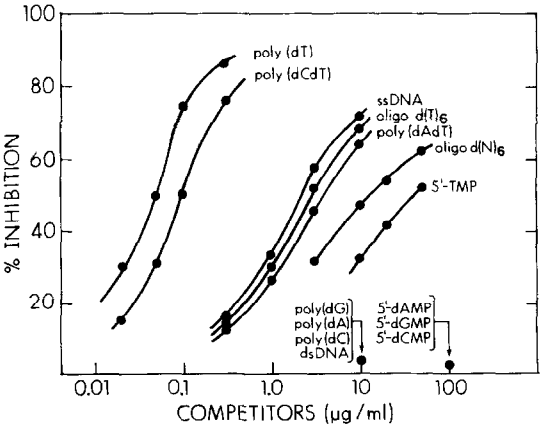

FIGURE 1: Competition-Inhibition ELISA. Inhibition of binding of affinity purified TMP-reactive autoantibodies to immobilized ssDNA by designated mono-, oligo-, and polynucleotide competitors. Details of these assays are described in Methods. Control assays (without competitors) yielded an absorbance (A405) value of 0.65 ± .02 units.

Table II. Inhibition of Binding of TMP-Reactive Autoantibodies to Immobilized ssDNA by Soluble Oligonucleotides [a]

No. Nucleotide Residues per Oligonucleotide	Concentration (ug/ml) of Competitor Required for 50% Inhibition					
	(dA)n	(dG)n	(dC)n	(dN)n	(dT)n	[(dN)n/(dT)n]
No. 1 = 1	>100	>100	>100		48	
2				101	22	[4.6]
4	> 50	> 50	> 50	32	8.1	[4.0]
6				12	3.0	[4.0]
8	> 50	> 50	> 50		1.3	
10				2.9	0.7	[4.1]
15					0.2	

[a]Data obtained in a manner identical to those presented in Figure 1 and extrapolated to determine concentration of each competitor required for 50% inhibition. (dN)n are mixed base oligonucleotides of defined size (n = 2, 4, and 6) generated by DNase I digestion of DNA and isolated by ion-exchance chromatography (14). (dN)10 was synthesized using phosphoramidite chemistry (DNA synthesizer, Applied Biosystems Model 380B).

continuous tracts of TMP residues (oligo (dT)n) are significantly more antigenic than their mixed base counterparts as well as a single TMP molecule. This assumption was verified by a second series of competition-inhibition studies which utilized various sequence- and size-defined oligonucleotides. These results are presented in Table II and report the concentration necessary for each competitor to inhibit anti-TMP antibody binding by 50%. First note that mono- and oligonucleotides (4- and 8- mers) lacking TMP were unable to achieve 50% inhibition at the highest concentration tested (i.e., 50 - 100 µg/ml). Second, the mixed-base oligonucleotides (oligo (dN)2,4,6) were 4-fold less inhibitory than their comparably sized oligo (dT)n counterparts. This finding is of interest when one considers that the former oligonucleotides contained approximately 4-fold less TMP residues than the latter. Most noteworthy was the finding that the quantity of competitor required to achieve 50% inhibition decreased with increasing oligonucleotide length for both (dN)n and (dT)n, ie, antibody affinity for oligonucleotides increases with antigen length.

SUMMARY AND CONCLUSIONS

The polyclonal nature of a diverse population of serum autoanti-DNA antibodies makes it difficult to obtain meaningful information regarding individual specificities. To circumvent this problem we have fractionated and purified these immunoglobulins into specific subsets based upon their reactivity with nucleotides. Utilizing this fractionation scheme we have previously determined that GMP-reactive autoantibodies recognize oligo (dG)n sequences (12). Here we extend our

observation to TMP-reactive autoantibodies. Our results (Table 1, Figure 1) indicate that oligonucleotides comprised entirely of TMP residues are preferred antigens and that antibody affinity to oligo (dT)n sequences increased with oligonucleotide length (Table II).

It is important to emphasize that these studies were derived from sera and thus reflect specifities belonging to a polyclonal population of TMP-reactive antibodies. We believe such results are more representative of serum autoantibodies relative to those based exclusively on selected monoclonal preparations (see ref. 2). One distinct advantage of the latter, however, is the absence of multiple specificities inherent in polyclonal preparations. Indeed such polyclonicity precludes direct assessment of specificity and requires adoption of fractionation procedures. While both protocols possess unique advantages, perhaps most significant is when data derived from each are in accord. In this regard various monoclonal antibodies derived from NZB/NZW mice demonstrate marked specificity not only for poly (dT) and ssDNA but for oligo (dT)n sequences as well (7,9,15). Further assessment of these monoclonals revealed marked increases in their relative affinities towards oligo (dT)n haptens as a function of oligonucleotide length (n=1-4,6,8,10, and 18) i.e., data in accord with that reported in Table II for polyclonal TMP-reactive autoantibodies.

Collectively these findings indicate that affinity purified TMP-reactive antibodies are monospecific for the thymine base moiety. Further, this base dependency is significantly enhanced by oligonucleotide length and ultimately results in an antibody population that can be defined as possessing a base (thymine)- dependent oligonucleotide specificity. The antigenicity associated with oligo (dT)n haptens should prove useful in selecting appropriate tolerogens for suppression of specific autoantibody populations (16).

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